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DOI:

[10.1016/j.pupt.2018.01.004](https://doi.org/10.1016/j.pupt.2018.01.004)

*Document Version*

Publisher's PDF, also known as Version of record

[Link to publication record in King's Research Portal](#)

*Citation for published version (APA):*

Lexmond, A. J., Singh, D., Frijlink, H. W., Clarke, G. W., Page, C. P., Forbes, B., & van den Berge, M. (2018). Realising the potential of various inhaled airway challenge agents through improved delivery to the lungs. *Pulmonary Pharmacology and Therapeutics*, 49, 27-35. <https://doi.org/10.1016/j.pupt.2018.01.004>

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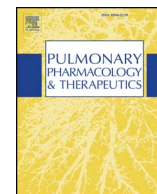
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## Realising the potential of various inhaled airway challenge agents through improved delivery to the lungs

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### ARTICLE INFO

#### Keywords:

Bronchial challenge test  
Bronchial hyperresponsiveness  
Asthma  
Drug delivery  
Delivery method optimisation  
Delivery method standardisation

### ABSTRACT

Inhaled airway challenges provoke bronchoconstriction in susceptible subjects and are a pivotal tool in the diagnosis and monitoring of obstructive lung diseases, both in the clinic and in the development of new respiratory medicines. This article reviews the main challenge agents that are in use today (methacholine, mannitol, adenosine, allergens, endotoxin) and emphasises the importance of controlling how these agents are administered. There is a danger that the optimal value of these challenge agents may not be realised due to suboptimal inhaled delivery; thus considerations for effective and reproducible challenge delivery are provided. This article seeks to increase awareness of the importance of precise delivery of inhaled agents used to challenge the airways for diagnosis and research, and is intended as a stepping stone towards much-needed standardisation and harmonisation in the administration of inhaled airway challenge agents.

### 1. Introduction

Inhaled airway challenges are a key tool in the study and diagnosis of obstructive lung diseases. Bronchial challenge tests that measure bronchial hyperresponsiveness (BHR) of the airways have established applications in the clinic, where they are used to rule out or confirm a diagnosis of asthma [1,2]. Inhaled airway challenges can also be used to study disease mechanisms and symptoms other than BHR, either by varying the outcome measure (e.g. inflammation measured by exhaled nitric oxide or inflammatory cell count) or the stimulus (e.g. allergen or endotoxin). The various airway challenges thereby allow the monitoring of disease activity and effectiveness of treatments [3], and they can provide a robust disease model in early phase clinical trials [4,5]. Given the reliance on inhaled airway challenges in respiratory medicine, there is surprisingly little standardisation of techniques or guidance regarding the administration of different test agents. In this article we consider various challenge agents and discuss the importance of standardisation and harmonisation of their administration methods.

Historically, bronchial challenge tests have been developed to measure BHR by means of spirometry and the change in forced expiratory volume in 1 s (FEV<sub>1</sub>) is still considered the primary outcome measure in the recently published technical standard on methacholine challenge testing [6]. However, whether FEV<sub>1</sub> is the most appropriate outcome measure is subject of debate and a recent study points out that the change in effective specific airway conductance (sG<sub>eff</sub>) measured with body plethysmography actually has a much larger diagnostic value than FEV<sub>1</sub> for the challenge agent methacholine [7]. Other techniques that can be used to measure airway function after provocation include forced or impulse oscillometry (airway resistance) [8] and multiple breath nitrogen washout (ventilation heterogeneity) [9]. The relative value of these techniques in challenge testing is beyond the scope of this paper. In this review we will refer to the outcome measure in a general way in appreciation of this on-going discussion.

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## 2. Inhaled airway challenge agents

Bronchial challenge testing can be performed with a wide range of stimuli, with selection of a particular agent depending on the aim of the test. BHR can be measured using stimuli that have either a direct effect on airway smooth muscle (ASM) (e.g. methacholine or histamine) or an indirect effect where the inhaled agent stimulates inflammatory or neuronal cells (e.g. mannitol, bradykinin or AMP) [10]. Furthermore, certain agents can be used that trigger other (patho)physiological mechanisms in the airways (e.g. endotoxin-induced inflammation or allergen-induced responses in allergic subjects), possibly accompanied by BHR in susceptible subjects.

### 2.1. Direct challenge agents

The most commonly used stimulus in bronchial challenge testing is the direct-acting stimulus methacholine, a synthetic analogue of the neurotransmitter acetylcholine that acts as an agonist on muscarinic  $M_3$  receptors on ASM cells. Histamine, an agonist for the histamine  $H_1$  receptors on ASM cells, can also be used, although this compound is associated with more systemic side effects such as flushing and headache due to vasodilation [11]. When a direct-acting stimulus is used the test generally has a high sensitivity for asthma, meaning that the majority of asthma patients will respond to this stimulus, and the responsiveness increases with the severity of lung disease [1]. However, the specificity is poor since healthy subjects also respond when the dose is high enough; they are just less sensitive and less reactive to the stimulus than asthmatic subjects (Fig. 1). Even though cut-off values for healthy and hyperresponsive individuals have been agreed upon [1,6,11], these values can still be considered as quite arbitrary due to the many factors affecting lung deposition of the stimulus, such as the patient's breathing pattern or the presence of emphysema, inflammation, mucus deposition and/or oedema. Moreover, it is becoming clear that methacholine can miss newly diagnosed asthmatic subjects whose symptoms are mild and whose lung function is excellent, but who demonstrate asthma in terms of significant exercise-induced bronchoconstriction [12]. However, the use of a different predefined threshold value for the outcome measure in methacholine challenge as compared to exercise-induced bronchoconstriction (*i.e.*, 20% reduction in  $FEV_1$  in the former versus 10–15% reduction in  $FEV_1$  in the latter) may explain, at least in part, such discrepancy. Additionally, testing with methacholine does not allow for absolute differentiation between patients with asthma or COPD, or indeed other diseases such as allergic rhinitis [13–16].

### 2.2. Indirect challenge agents

In the search for stimuli that produce responses through mechanisms that better reflect the underlying disease pathology, indirect

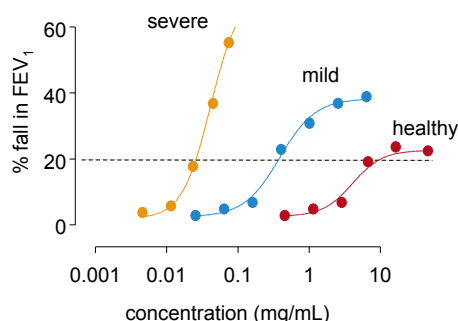


Fig. 1. Dose-response curves to inhaled methacholine in a healthy, mild-asthmatic, and severe-asthmatic subject, showing both the leftward shift of the curve (hypersensitivity) and steeper slope (hyperreactivity) that characterise BHR. Reproduced with permission from the European Respiratory Society [1,117].

challenges have been introduced, which exert their effects on intermediary cells involved in the asthmatic response, rather than acting directly on ASM. Most indirect stimuli evoke a heterogeneous response by affecting multiple pathophysiological pathways [3]. Especially in the 1980s and 1990s many different potential indirect stimuli have been investigated, which have been reviewed comprehensively by Van Schoor et al., first in 2000 [10], and subsequently updated in 2005 by the same authors [17]. In 2003, a European Respiratory Society (ERS) Task Force published their recommendations on the use of indirect stimuli in diagnosis and monitoring of asthma [3].

Indirect stimuli can be sub-classified as physical or pharmacological stimuli. Physical stimuli induce airways obstruction without acting on specific receptors, exemplified by exercise-induced bronchoconstriction or that induced by “fog” challenges with distilled water or hypotonic aerosols [18,19]. Exercise induces dehydration of the airway epithelium, resulting in an increased osmolarity of the airway lumen and subsequent release of mediators from mast cells and activation of sensory nerves [20]. This process is mimicked during challenge with hyperosmolar aerosols [21,22]. Pharmacological stimuli induce airways obstruction secondary to the activation of intermediary cell types, such as inflammatory, epithelial, or neuronal cells, or combinations of these. The effects of indirect agents depend on the specific cells and receptors involved [10], but many of the stimuli used are known to activate sensory nerves, for example bradykinin, sulphur dioxide and adenosine (reviewed in Ref. [23]). Some indirect stimuli are endogenous compounds known to be released during airways obstruction, such as adenosine, AMP, tachykinins and bradykinin [24–31]. Another group of indirect-acting stimuli is comprised of sulphur-containing compounds, which originated from the observation that sulphur dioxide, a common air pollutant, and sulphites used as preservatives in food processing, may induce bronchoconstriction in susceptible subjects through activation of sensory neuronal pathways [32,33]. However, lack of reproducible test outcomes has led to discontinuation of studying several of these stimuli (sulphur dioxide, sodium metabisulphite, bradykinin and tachykinins) and focus has predominantly shifted to the most easily and widely applicable indirect stimuli, mannitol (physical) and AMP/adenosine (pharmacological).

### 2.3. Allergen challenge

The preceding “non-specific” bronchial challenge tests are targeted to mechanisms that are thought to be intrinsic to the underlying hyperresponsive state of the airways in subjects with asthma. In contrast, so-called “specific” airway challenges can be used to assess the airway responsiveness to sensitising agents, such as aeroallergens or occupational agents. In allergic subjects, following sensitisation to an allergen, minute quantities of that allergen are sufficient to cause an immediate IgE-mediated early asthmatic response (EAR). In approximately 50% of positive allergen challenges a recurrence of airflow obstruction occurs between 3 and 8 h after allergen exposure, the so-called late asthmatic response (LAR) [34], which is associated with airway inflammation and in some patients can be associated with an increase in BHR to agents like methacholine [35–37]. Often-used outcome measures for inhaled allergen challenge are a > 15% decrease in  $FEV_1$ , > 50% decrease in specific airway conductance, or > 100% increase in specific airway resistance compared to baseline [38]. Additional outcome measures can be the change in non-specific BHR to e.g. methacholine, or the occurrence of airway inflammation expressed as increase in sputum eosinophils or exhaled nitric oxide [38]. Inhaled allergen challenge has also been widely used as a model to assess the efficacy of novel therapeutic interventions [39–49].

### 2.4. LPS challenge

Another interesting application of the inhaled airway challenge test concept is provocation with lipopolysaccharide (LPS), also called

endotoxin, a major component of the outer membrane of Gram-negative bacteria that induces fever and inflammation upon systemic exposure [50]. Aerosolised endotoxins are present ubiquitously in the environment in concentrations that do not elicit immune responses. However, in certain aerosols, like tobacco smoke and organic dusts, concentrations can be high enough to induce responses in the lungs [51]. Inhalation of endotoxin has been associated with lung inflammation, most notably neutrophilia [52–54]. It can further lead to bronchial obstruction in people with asthma or other forms of BHR [55,56]. Interestingly, it has recently been reported that LPS can elicit BHR through a mechanism involving cholinergic transmission [57].

Airway challenge with a nebulised LPS solution has been used mainly to study neutrophilic inflammatory processes in the lungs and has been developed into a challenge model to investigate the effect of novel anti-inflammatory drugs under development for the treatment of diseases that are associated with neutrophil infiltration into the lungs, particularly COPD and severe asthma, as this challenge is not sensitive to treatment with glucocorticosteroids [5,58–61]. This model has also been used for early proof-of-concept and dose-ranging studies for novel drugs for the treatment of respiratory diseases in healthy volunteers [58,62].

### 3. General considerations for challenge delivery to the lungs

Despite the wide array of challenge test agents, there are relatively few methods used for their administration. Hence various aspects need to be considered that apply to airway challenge methodology in general, irrespective of the agent that is used.

#### 3.1. Delivered dose

Inhaling a challenge agent usually leads to a dose-dependent response in the lungs. For challenges that induce BHR, the minimal dose required to obtain a response, as well as the slope of the dose-response curve, varies amongst patients and defines the severity of their BHR [1]. The aim of such bronchial challenge tests is to induce a pre-defined degree of bronchoconstriction without risking a response that is too severe, meaning that the dose of the stimulus requires titration. For this reason, ascending dosing protocols have been developed in which the stimulus is administered by nebulisation of ascending (usually doubling) concentrations or doses, after each of which the lung function is measured [1,6,11,63,64]. The concentration or dose is gradually increased and the test continues until a predefined threshold value for the outcome measure is obtained. The test result is negative when the threshold value is not reached after administration of the top dose.

This methodology has been initially developed for direct stimuli [65], but indirect-acting stimuli are generally administered following similar dosing protocols [3], although different concentrations (or doses) may be used to accommodate differences in potency, as shown in

Fig. 2. For AMP for example, a 16-fold lower potency has been reported compared to methacholine [66]. Allergen exposure should be increased gradually either by extending the duration of the exposure or increasing the concentration in order to prevent severe acute reaction [38]. For the same safety reason, allergen dose increments should have longer time intervals (10–15 min) compared to direct or indirect challenge methods (< 5 min). Due to their specific mode of action, as little as a few ng can suffice to elicit the airway response. Endotoxin challenge on the other hand is usually administered by nebulisation of a fixed dose in the µg-range.

It is clear that due to these differences in dosimetry, the various challenges require different methods for administration. However, currently no universally standardised methodology is used in the majority of cases to deliver these different agents. It is important to realise that this may have important implications that are now largely neglected by the field.

#### 3.2. Administration by nebulisation

Most challenge agents are administered by nebulisation, except for mannitol and an investigational formulation of adenosine that are both administered as a dry powder (see below). Preparing nebuliser formulations can be very straightforward, which is the main reason why nebulisers are often used for off-label or investigational drugs and non-medicinal compounds, like many of the agents used for challenge testing. However, it has to be carefully evaluated whether the formulation affects nebuliser performance (in terms of droplet size and output rate) and, in the case of more complex molecules, whether the nebulisation process leads to degradation of the agent (e.g. allergens of biological origin).

Two standardised dosing protocols have been published for administration of methacholine by nebulisation [6,11]. The two methods have different pros and cons, and the choice of method has been left to the preference of individual investigators/clinicians. The dosimeter method involves five deep and slow inhalations, which allows for accurate quantification of the administered dose, thereby making this method suitable for studies that require administration of an exact dose, such as LPS challenge studies or allergen challenges using a bolus dose. It has been claimed, however, that such deep breaths have broncho-protective and bronchodilatory effects per se [67–69], which may therefore interfere with an accurate interpretation of the test result. In the newest technical standard on methacholine challenge testing a deep-breath method is therefore not recommended [6]. The other method is the tidal breathing method in which the stimulus is inhaled during a specified time of calm, tidal breathing, although the patient's inhalation flow rate is generally not controlled. This more shallow way of inhaling does not evoke bronchoprotective and bronchodilatory mechanisms, but could result in different deposition patterns of the aerosolised challenge compared to the five-breath dosimeter method, since penetration of the aerosol into the more distal airways is dependent on the mixing of old and new air in the lungs. Moreover, the total amount that is inhaled depends on the inspiratory cycle of the subject, but also on the output rate of the nebuliser. In the first universal (American Thoracic Society) guidelines on methacholine challenge testing, the output rate had therefore been standardised at 0.13 mL/min (based on the output rate of the Wright nebuliser that was commonly used for challenge testing), regardless of the nebuliser that was used [11]. However, adjusting the jet pressure to obtain this output rate may have detrimental effects on the droplet size distribution. As an example, this has been shown for the SideStream nebuliser (Philips Respironics), where the median mass aerodynamic diameter (MMAD) increased from 5.1 µm to 8.5 µm when the jet pressure was reduced from 1.5 bar (manufacturer's specifications) to 0.5 bar to reach the required output rate of 0.13 mL/min [70]. To prevent such unforeseen changes, a better strategy is to control the total administered volume of the solution containing the challenge agent by altering the nebulisation time rather

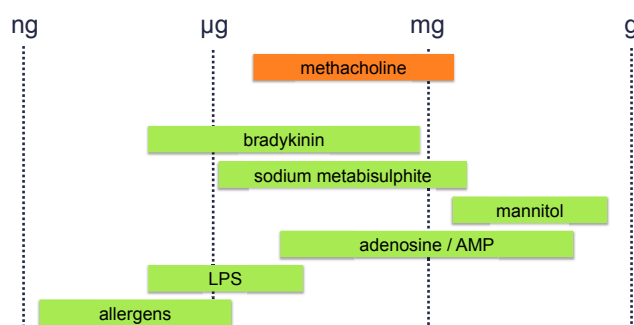


Fig. 2. Dose ranges of various inhaled airway challenge agents. Allergens and LPS can also be expressed in biological units and endotoxin units respectively.

than using an output rate of the nebuliser outside of the manufacturer's specifications, which is now discussed in the recently published European Respiratory Society technical standard on methacholine challenge testing [6].

In addition to the effect of jet pressure on droplet size, the type of compound and its concentration may affect the droplet size distribution as well. For AMP, which has a dose range exceeding that of methacholine, it has been shown that the increased viscosity of the more concentrated solutions resulted in a large shift in the aerosol droplet size distribution [70]. In this study, a decrease in MMAD of almost 50% was measured at the highest AMP concentration (320 mg/mL) compared to saline and the lowest AMP concentrations. These results indicate that applying methods developed for a certain stimulus and a certain device cannot simply be used for other compounds or in other situations without verifying their suitability.

A third factor that should be accounted for is the evaporation of solvent (water) during nebulisation. The driving force for evaporation is saturation of the outgoing air with solvent [71], which leads to an increase in concentration of the remainder of the solution in the nebuliser cup [70,72]. Importantly, evaporative water losses lead to an overestimation of the administered dose when the output rate is measured gravimetrically [6]. Evaporation is an endothermic effect and the energy needed for this process is drawn from the solution, resulting in a temperature drop in the nebuliser solution that in turn may affect the output rate of the nebuliser [72]. Calibration of the nebuliser output rate should therefore be performed under precise operating conditions. Newer jet nebulisers and especially vibrating mesh nebulisers exhibit much lower amounts of evaporative loss [6], but still it is preferable to measure output rate by means of filter measurements (collection of the active compound) rather than gravimetrically. Such data should either be provided by the manufacturer or can be obtained in a pharmaceutical lab specialised in inhaled drug delivery.

In contrast to methacholine, there are no universal standardised protocols for other challenge agents, although recommendations have been made for similar ascending administration protocols [3].

### 3.3. Aerosol deposition and distribution in the lungs

For optimal efficacy and discriminatory power, inhaled medical aerosols should achieve maximal delivery to, and deposition in the target area in the lungs. Bronchial challenges that measure bronchoconstriction should be targeted to the proximal part of the bronchial tree, where the effects of ASM contraction are most pronounced. This means that the requirements for aerosol particle size are quite easily met, since an MMAD of roughly 3–5  $\mu\text{m}$  should generally suffice, especially at tidal breathing. Aerosols with particles in this size range have the additional benefit of increased deposition efficacy, resulting in a higher total lung dose compared to particles smaller than 1.5  $\mu\text{m}$  [73]. It has indeed been found that aerosols with MMADs of 3 and 5  $\mu\text{m}$  result in a lower methacholine PC<sub>20</sub> compared to those with an MMAD of 1  $\mu\text{m}$  [74], which can be attributed to a combination of greater lung deposited dose and targeting to the proximal airways.

It could be reasoned that challenges that act on inflammatory processes should be targeted more distally, as inflammation occurs throughout the lungs. However, investigations into the effects of particle (droplet) size on airway responsiveness to AMP have thus far been inconclusive due to a high number of non-responders to small-particle AMP, which could be explained by a higher exhaled fraction or the discrepancy between deposition in the peripheral airways and an outcome measure of the more central airways (FEV<sub>1</sub>) [75]. For allergens, significant effects of particle size on the response to cat and mite allergen have been found, with larger particles (around 10  $\mu\text{m}$ ) being more effective in inducing the immediate response [76,77]. A study investigating the effect of particle size on responses to endotoxin found a greater inflammatory response at the bronchial and systemic level when challenged with larger particles, although it could not be

concluded whether this was due to regional distribution differences or the higher total lung dose [78].

### 3.4. Patient-related factors affecting aerosol deposition

Patient-related factors, such as the size and morphology of the oropharynx and bronchial tree, and the severity of lung disease can also affect aerosol deposition patterns [79]. Additionally, inhalation flow rate has an important influence on the site of deposition [80]. A higher flow rate shifts deposition to the higher airways at the cost of peripheral deposition. The patient's flow rate should therefore be adjusted to the type of delivery device being used to prevent loss of aerosol through deposition in the throat. Newer delivery systems that provide electronic control over the inhalation flow rate and volume (e.g. APS Pro system, see section 4.1, and AKITA) can provide better control over the delivered dose and deposition in the lungs [81–83].

Current medication use of patients undergoing a bronchial challenge has to be accounted for as well, since these treatments are intended to reduce or prevent the symptoms evoked during the challenge. To prevent possible confounding effects on the test outcome, lung medications have to be withheld for a specified time prior to execution of the test. The duration of withholding is dependent on the mechanism of action of the drug and ranges from a few hours for short-acting beta-agonists up to a few days for long-acting anticholinergics or even weeks for ICS (depending on the challenge agent and the aim of the challenge test).

## 4. Optimisation and standardisation of challenge delivery per agent

Both optimisation and standardisation of challenge methods by different agents are urgently needed, in order to address the issues identified in the preceding section and make scientific progress towards more precise and rigorously controlled diagnostic procedures. Some efforts have already been undertaken in this regard, for example with the mannitol test (Aridol/Osmohale; see section 4.2).

### 4.1. Methacholine

Soon after publication of the first universal guidelines for methacholine challenge in 2000, which recommend both the dosimeter method and tidal breathing method, studies began to appear that investigated the comparability of the two dosing protocols. The first study reported similar results (geometric mean PC<sub>20</sub> 1.8 mg/mL for tidal breathing vs. 1.6 mg/mL for dosimeter). However, the authors compared different dosing protocols (twofold vs. fourfold increases in concentration for tidal breathing and dosimeter respectively) [84]. Cockcroft et al. addressed this disparity by comparing identical dosing regimens (doubling concentrations) and found that the tidal breathing method, which exposes the subject to twice as much aerosol at each concentration, resulted in a PC<sub>20</sub> that was 1.6 (PC<sub>20</sub> < 1 mg/mL) to 2.1-fold (PC<sub>20</sub> > 1 mg/mL) lower compared to the dosimeter method [85]. This difference between subjects with mild and severe hyperresponsiveness has been suggested to be explained by bronchodilator and/or bronchoprotective effects of the inhalation manoeuvre adopted in the dosimeter method [85,86]. Prieto et al. reported a difference of 0.78 doubling concentrations, with dosimeter values being higher than tidal breathing values, but found similar values for slope and level of plateau of the dose-response curve [87]. Acknowledging the difference in administered volume, they performed another study in which they administered the same volume of challenge. Still an average difference in PC<sub>20</sub> of 0.9 doubling concentrations was reported [88]. However, when looking at the individual subjects it can be seen that this difference was mainly caused by a higher number of non-responders when using the dosimeter method (Fig. 3). This may actually indicate that in some subjects with (mild) asthma the bronchodilatory effect of deep



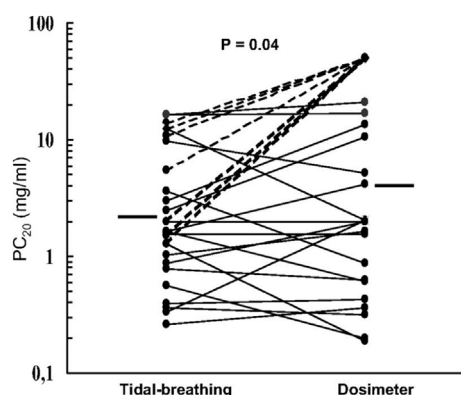


Fig. 3. Comparison of tidal breathing and dosimeter methacholine  $PC_{20}$  in 27 subjects with suspected asthma. The dashed lines indicate seven subjects in whom no dosimeter  $PC_{20}$  was obtained. Reproduced with permission from Ref. [82].

inhalations can indeed effectively counteract any bronchoconstriction induced by methacholine, as suggested by Cockcroft et al. [85]. Indeed deep-breath methods have now been excluded from the new technical standard on methacholine challenge for this reason [6].

More recent investigations into methacholine challenge method optimisation focused on the delivery systems used, as the nebulisers recommended in the first guidelines [11] had become obsolete [89]. The Aerosol Provocation System (APS) Pro (CareFusion) is especially noteworthy in this respect. The APS Pro system is a computer-controlled nebuliser system specifically developed for bronchial challenge testing with methacholine and can be integrated with other CareFusion systems for spirometry, impulse oscillometry, respiratory resistance and body plethysmography depending on the outcome measure of choice. The dosimeter method is the preferred method, although the system can also be used for the tidal breathing method. The  $PD_{20}$  from a pulse of aerosol of a single methacholine concentration was found to correlate well with the  $PC_{20}$  obtained with a standard dosimeter test [90]. *In vitro* studies indicate that these new delivery systems often have a higher output rate than the systems recommended in the first guidelines, thus a faster delivery of the challenge, which should be controlled for in terms of exposure time [70,89].

The plethora of available nebuliser systems (with variable output rates) introduces new concerns regarding equivalence of the test results obtained with different systems. Interestingly, it has been found that no differences between test systems are found when the  $PD_{20}$  is calculated [91,92] instead of the  $PC_{20}$  [91,93], which has led to the recommendation to use the  $PD_{20}$  as the end-point in the new technical standard [6]. On a different note, using more efficient nebulisers also introduces a risk of extreme individual responses to methacholine aerosols. Patients who are highly responsive to methacholine may experience large drops in  $FEV_1$  when the full dose is presented to them in a shorter time, which should be accounted for by thorough safety assessment of using these more efficient nebulisers.

#### 4.2. Mannitol

Bronchial challenge with mannitol has been developed to overcome technical difficulties (i.e. the need for filters and scales to determine the administered volume) encountered with bronchial challenge tests using hypertonic saline [20,94,95]. This agent has been shown to cause contraction of ASM through release of inflammatory mediators such as leukotriene  $E_4$  and prostaglandin  $D_2$ , which are thought to be released from mast cells [96]. BHR measured in response to inhaled mannitol is dependent on the presence of inflammation and can be reduced by ICS treatment [97,98]. The low sensitivity (59.8%) of the mannitol test compared with the clinical assessment determined in more than 500 subjects was attributed to ICS use by 75% of the diagnosed asthmatics

in this study, as this value greatly improved when ICS-users were excluded from analysis (to 88.7%) [95]. This finding supports the concept that mannitol responsiveness can be used to monitor ICS effectiveness [97,98] and highlights the growing appreciation that indirect challenge tests are useful for diagnosis and monitoring treatment of current asthma. Mannitol responsiveness – expressed as the provocative dose that causes a 15% decrease in  $FEV_1$  ( $PD_{15}$ ) – has been found to correlate well with responsiveness to bronchial challenge tests with other physical stimuli or AMP [99,100]. Bronchial challenge testing with mannitol may therefore be of particular use in diagnosis of asthma in elite athletes, who require an official diagnosis of asthma, but whose bronchoconstriction is hard to induce by exercise in a laboratory setting [101].

The mannitol challenge test is registered with various regulatory authorities worldwide and is currently the only fully standardised challenge method. The mannitol formulation consists of a spray-dried powder with an MMAD of around  $3.5\ \mu\text{m}$  that is inhaled with a simple capsule inhaler device. Benefits of this mannitol test are that it comes in a standardised kit that does not need any special equipment, and that it is relatively easy to perform. However, a drawback of mannitol challenge is the deep inhalation-dependent modality of powder administration, which has been suggested to counteract bronchoconstriction as discussed in section 4.1. Moreover, the quantity of powder that needs to be inhaled is large in comparison to other agents, up to a cumulative dose of 635 mg, as a consequence of its mechanism of action (i.e. increasing the osmolality of the lung lining fluid). This in combination with the low resistance device, and hence a high inspiratory flow rate, can result in cough through a mechanical cough reflex due to oropharyngeal deposition of the mannitol [102]. In a phase III study investigating the safety and efficacy of inhaled mannitol as a bronchial challenge test, cough occurred in 535 of 592 (of whom 91 were non-asthmatic) subjects. In some cases, cough was so severe that the test had to be delayed (one in seven subjects), or even ended prematurely (one in 100 subjects) [95]. Although cough does not occur exclusively in subjects with asthma, it has been demonstrated that cough in response to inhaled mannitol is associated with asthma [103], which would be interesting to elucidate further. To which extent the occurrence of (severe) cough is diagnostic for asthma and to which extent it is due to oropharyngeal deposition of mannitol could be investigated by provoking subjects with the same mannitol formulation, but using a high-resistance inhaler device and controlled slow inhalation to minimise throat deposition.

#### 4.3. Adenosine

Other efforts towards optimisation have been undertaken with inhaled adenosine. Adenosine and its precursor AMP have been the subject of a considerable amount of research in respiratory medicine since the early 1980s. Adenosine, a purine nucleoside involved in many biological processes, is considered a pro-inflammatory mediator in asthma [104] as it is thought to induce mast cell degranulation, a process mediated through the  $A_{2b}$  receptor, leading to contraction of ASM and most notably airway eosinophilia [105,106]. More recently a role for  $A_1$  receptors has been implicated in the contraction of ASM from subjects with asthma induced by adenosine [107], suggesting that adenosine triggers bronchoconstriction through both inflammatory and neuronal pathways.

Historically, AMP has been used instead of adenosine because of its much higher aqueous solubility, which is required for nebulisation, and it is generally assumed that AMP is converted *in vivo* to adenosine instantaneously by endonucleotidases when it comes in contact with lung lining fluid [10]. However, because of the above mentioned effects of high AMP concentrations on aerosol particle size produced by nebulisation [70], an adenosine dry powder challenge test has been developed that consists of simple spray-dried formulations containing pure adenosine or adenosine diluted with lactose, which so far have

only been administered with an investigational inhaler device. With this inhaler, the entire dose range of adenosine (0.04–80 mg) was consistently delivered in the first proof-of-concept studies that have been performed with this formulation [108–110]. So far, these studies justify the chosen dose range for adenosine and indicate that the response rate and thus diagnosis of asthma can be improved by the administration of the higher doses that are possible with the powder formulation. These findings now have to be complemented by studies in healthy subjects and in subjects with lung diseases other than asthma to determine the specificity and sensitivity of this test. Since the test concerns a powder for inhalation, any effects on bronchoconstriction of the deep-inhalation dependent administration should also be considered for this adenosine challenge test.

#### 4.4. Occupational agents and allergens

For occupational agents, the suspected causative agent should be delivered in the same conditions that it is found in the workplace in terms of physical and chemical properties in relevant concentrations [38]. It is now recognised that there are a large variety of occupational agents and allergens and therefore a handbook has recently been prepared that summarises the delivery methods for the most commonly used agents (see online supplement to [38]). This handbook provides an excellent start towards harmonisation of specific inhaled challenges, although it could benefit from inclusion of recommendations on nebulisers for those agents that are administered following a tidal breathing or dosimeter method.

Allergens are administered in very low doses compared to the nonspecific bronchial challenges. Nebulisation can therefore in general be considered a suitable administration method, provided the stability of the agent is checked during storage and upon administration, particularly for more complex molecules (e.g. antigens). Chemical stability issues upon storage arise when an agent is sensitive to degradation reactions (e.g. oxidation, hydrolysis), as these occur faster in aqueous conditions than in the dry state. Additionally, stability can become an issue when the stresses induced by the nebulisation process itself may damage the material(s) (e.g. proteins) in the formulation [111,112].

#### 4.5. LPS

LPS is very stable and can withstand high temperatures and strong shear forces. It can be kept in solution for up to a month. However, LPS adheres readily and strongly to surfaces such as glass, for example to the vial in which it is stored. Extra care (e.g. rigorous vortexing) should therefore be taken in the preparation of the nebuliser solution. Additionally, endotoxins from different sources can have a different biological activity (potency). Studies report the use of different sources of LPS and different doses, ranging from 0.5 to 100 µg [5,52–54,59–62]. However, expressing dose in units of weight has little value because of

the different potencies. Variability in the dose delivered to patients is further increased by differences in administration method. Both dosimeter and tidal breathing methods have been used to date and the differences between devices and inhalation manoeuvres inevitably result in differences in the delivered and deposited doses.

The lack of control of dose in terms of potency, in addition to differences in administration methods, complicates the comparison of studies performed over a period of about 30 years. Studies have been performed in healthy [5,51–54,58–62,113] and diseased subjects [55,56,114,115], in smokers [116] and non-smokers, but the potency of the LPS was often not reported. Although a promising disease model, standardisation of the dosing protocol and administration method should be established before LPS challenge can be accepted as a validated tool to be used more widely in drug development studies. Other issues that need to be addressed are the lack of dose-response studies performed in humans and uncertainty regarding why some people do not respond to LPS inhalation. To study the latter issues, it is imperative to know the exact dose of LPS that is delivered and its potency. The first steps forward should be to decide on a preferred administration method (i.e. slow deep inhalation) and performing a potency measurement of the LPS in the nebuliser solution to be used for administration.

## 5. Conclusions

Inhaled airway challenges are versatile tests that are relatively easy and cheap to perform. Classical bronchial challenge tests that assess BHR have proven their value in excluding or confirming a suspected diagnosis of asthma and have been shown to be useful for monitoring the disease and effectiveness of therapy. These tests can thus help in providing more accurate information to patient and prescriber as to how to treat an individual patient. As such, bronchial challenge tests can help in improving the individual patient's health through better treatment of their disease. In research and development, inhaled airway challenge can be applied even more widely, from studying disease mechanisms to investigating the effectiveness of new drugs. Careful selection of the challenge agent may provide significant benefits, in terms of both selecting suitable subjects (e.g. using response to a discriminatory challenge as an inclusion criterion) and addressing the research question. There is also a lot to be gained through optimisation and reporting of challenge test posology, especially to ensure the comparability of studies performed by different laboratories. In general all compounds described so far would strongly benefit from the development of defined inhalation systems that provide a reproducible and reliable deposition in the lungs and further standardisation of administration protocols as has been done for mannitol (Table 1). Improved delivery may also open doors for revisiting some challenge agents that have been used in the past, but were abandoned due to lack of reproducibility. Creating a “tool box” of well-characterised challenge agents with tailored delivery systems would provide a valuable tool for

**Table 1**  
Opportunities to improve the application of bronchial challenge testing.

| Issue                         | Need   |
|-------------------------------|--|
| Delivery method optimisation  | Control (and quantification) of delivered dose by optimising the production and administration of challenge agent aerosols with suitable aerodynamic size distributions. This will require the tailoring of delivery methods for each individual challenge agent.  |
| Standardisation               | International consensus on best practice.<br>Inter-lab comparisons to verify the reproducibility of standard methods.<br>Reporting of dose characterisation/validation for all research studies.   |
| Specific issues for mannitol  | Investigate the relative contributions of throat deposition and increased airway sensitivity to the occurrence of cough in asthma.   |
| Specific issues for adenosine | Compare responsiveness to dry powder adenosine challenge to responsiveness to nebulised AMP including the response of healthy subjects and patients with lung diseases other than asthma.<br>Determination of the specificity and sensitivity of dry powder adenosine bronchial challenge in patients with asthma. |
| Specific issues for allergens | Extension of existing guidelines [37] to include recommendations on nebulisers for those agents that are administered following a tidal breathing or dosimeter method.<br>Verification of chemical stability and tolerance to nebulisation on a case-by-case basis.  |
| Specific issues for LPS       | Control of test agent potency and reproducibility of delivery (dose and lung distribution).  |

studying and discriminating different airway diseases, but also for investigating mechanisms and novel treatments for affecting BHR.

## Disclosures

DS reports grants and personal fees from Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Glenmark, Johnson and Johnson, Merck, NAPP, Novartis, Pfizer, Takeda, Teva, Therevance and Verona, and personal fees from Genentech and Skyepharma, outside the submitted work. HWF reports that his employer receives royalties on the sales of the Genuair inhaler from AstraZeneca and royalties on the sales of the Novolizer inhaler from MEDA, outside the submitted work. HWF has a patent PCT/NL2004/000427 licensed to PureIMS, which involves the inhaler technology used for administration of the dry powder adenosine formulation. MvdB reports grants paid to the University from Astra Zeneca, TEVA, GSK and Chiesi, outside the submitted work. GWC was not affiliated with hVivo Ltd at the time of initial writing of the manuscript and declares that his involvement is purely on a scientific basis. AJL, GWC, CPP and BF declare that they have no competing interests.

## Author's information

AJL was holder of the C.W. Maplethorpe Postdoctoral Fellowship of the University of London during the writing of this work.

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